Hydrophilic Interaction Chromatography (HILIC)

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HILIC

“A method of recent attention”

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HILIC

… but not the universal answer.

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Objective - Outline

- **Overview - Why HILIC is used Advantages/Disadvantages**
- **How HILIC works**
- **Examples**
- **Method development**
- **Comments concerning use of HILIC**

Why HILIC Is Used

- **Retention of polar components**
- **Similar to NPLC but with aq mobile phase (General reversal of elution order from RPLC)**
- **Several different stationary phases available**
- **MS compatible**
- **May simplify sample prep**

… but there is always a price

- **Slower equilibration than RPLC**
- **Peak distortion with mp-sample solvent mis-match (*i.e.*, too much water in sample solvent)**
- **Poor retention of anions on silica**
- **Mechanism not well understood**

Additional Benefits, Comments

- ACN has low viscosity – allows high flow rates
- Van Deemter plot similar to RPLC
- Not truly "orthogonal" to RPLC, but dissimilar
  – not just inverse separation
  – complementary technique
- Type B Silica, high purity, less acidic
  – less active but more reproducible

Parameters to Choose

- Type of stationary phase
- Organic solvent concentration
- Type of buffer
- Buffer (salt) concentration
- pH
- Temperature

Typical Stationary Phases - Polar

- **Silica**
- **Amine**
- **Diol**
- **Amide**
- **Zwitterionic**

Some Typical Polar Stationary Phases

- Silica, Si-OH $\rightleftharpoons$ Si-$O^{(-)}H^{(+)}$
- Amine, -$(CH_2)_3$-NH_2
- Diol, -$(CH_2)_3$-O-CH_2-(CHOH)CH_2OH
- Amide, -$(CH_2)_n$-(CO)NH_2
- Zwitterionic, -$(CH_2)_n$-$N(Me)_2^{(+)}$-$(CH_2)_n$-$SO_3^{(-)}$

Typical Mobile Phase

- **Water /ACN**
- **Buffer**
- **pH**

Typical Mobile Phase

- **Water (at least 2- 3%, ~ 25%)/CAN**
  Solvent strength:
  Water > MeOH > EtOH > IPA > ACN

- **Buffer AmOAc, 5 to 20 mM**
  Buffer must be soluble in high CAN concentration

- **pH 3 – 8**

Mobile Phase

- **Need some water for hydration**
- **Water is “strong solvent”**
- **Increasing water, decreases retention**

Buffer

- Controls Ionization of Silica
- Controls Ionization of Analyte
- Ammonium acetate, formate – good solubility, “MS friendly”
- Phosphate - poor solubility at high % ACN

pH

- **Controls Ionization of Silica**
- **Controls Ionization of Analyte**
- **Organic solvent affects the actual [H^+]**

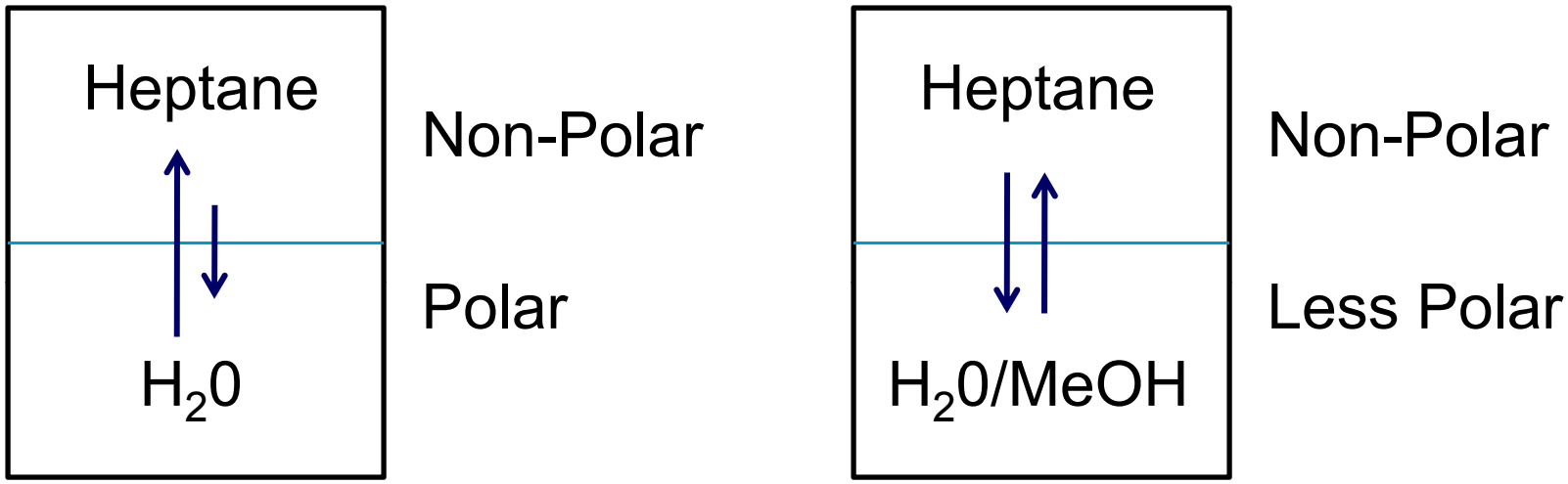
Temperature

- Higher Temperature Decreases Ret'n Time
- Higher Temperature Increases Column Efficiency (increases N)
- Lower Temperature May Increase Selectivity

Separation Mechanism

Retention in Reversed-Phase Separations: Mechanism Similar to Liquid-Liquid Extraction

In RPLC decrease retention by decreasing polarity of mobile phase



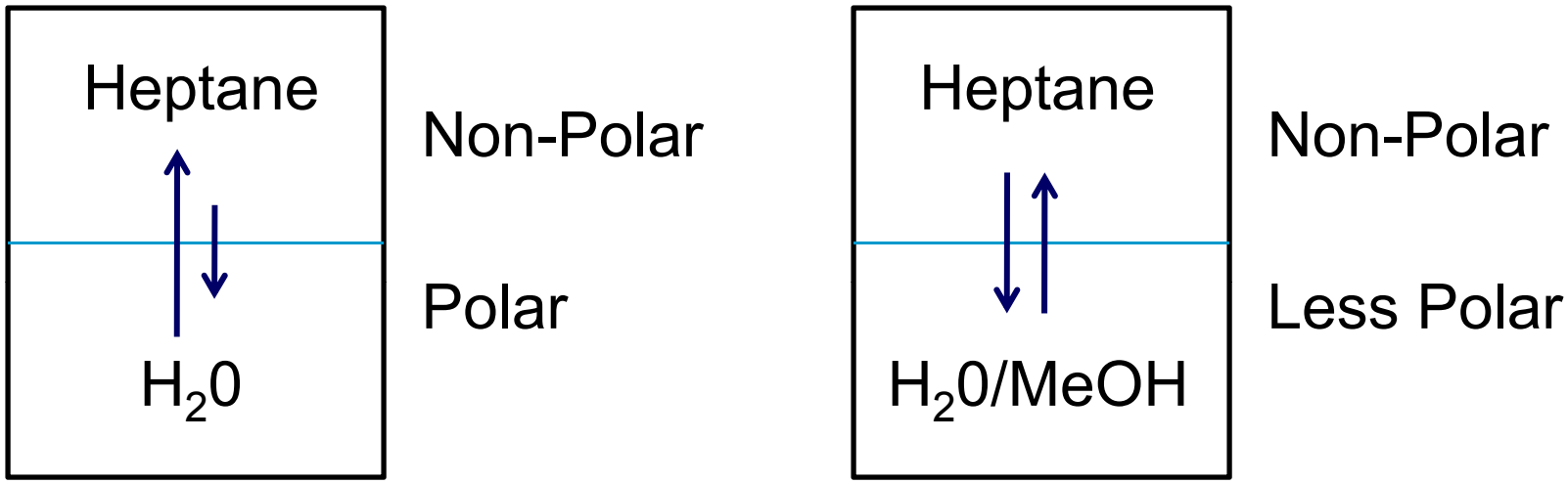
Solvent Polarity: H2O > MeOH, ACN > EtOH > IPA >> Heptane

Relatively non-polar analyte (*e.g.*, toluene) favors non-polar phase.

Decreasing polarity of aqueous phase increases affinity for analyte in H_2O/MeOH phase.

Retention in Reversed-Phase Separations: Mechanism Similar to Liquid-Liquid Extraction

In RPLC decrease retention by decreasing polarity of mobile phase



Mobile Phase Strength: H2O < MeOH, ACN < EtOH < IPA << Heptane

Relatively non-polar analyte (*e.g.*, toluene) favors non-polar phase.

Decreasing polarity of aqueous phase increases affinity for analyte in H_2O/MeOH phase.

Comparison of HILIC and RPLC

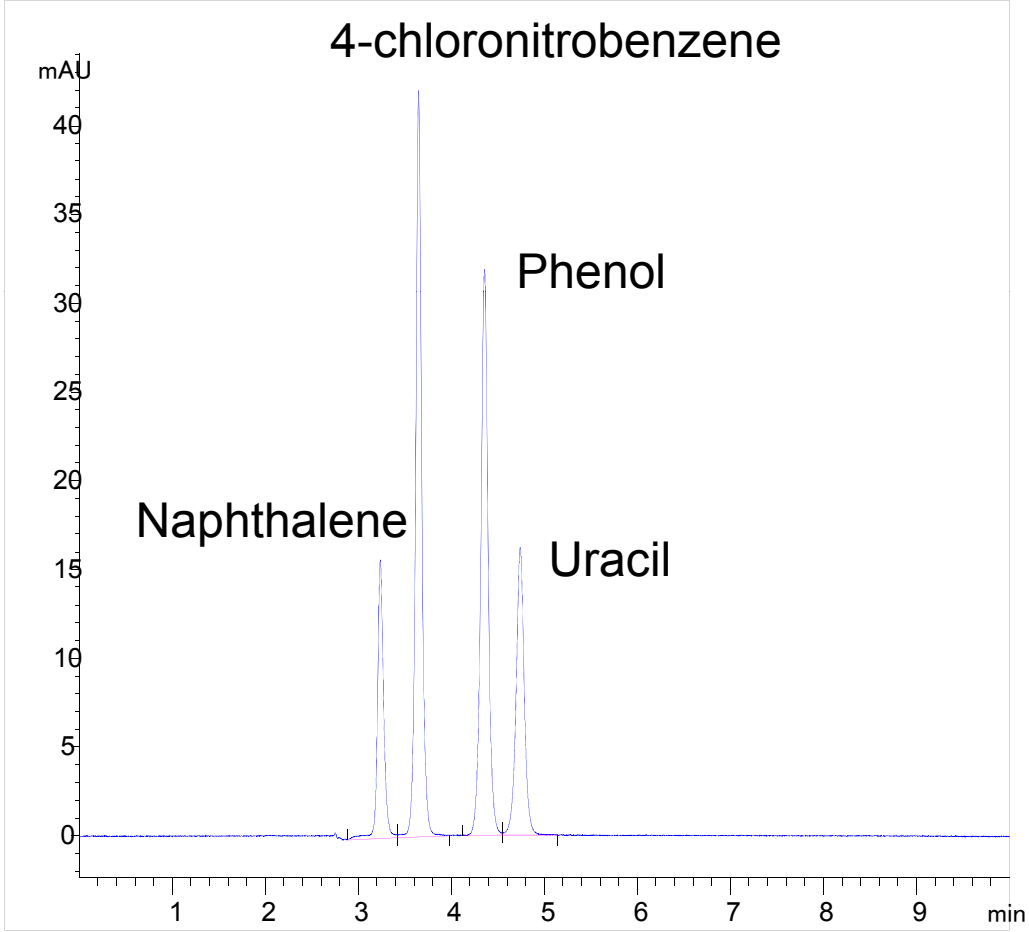
RPLC

- Non-polar stationary phase (*e.g.*, C18)
- Polar mobile phase (*i.e.*, H_20/MeOH, H_20/ACN, etc.)
- Decrease retention by *decreasing* polarity of mobile phase (*e.g.*, increase **ACN** in mobile phase to decrease retention)

HILIC

- Polar stationary phase (*e.g.*, silica)
- Polar mobile phase (*i.e.*, H_20/ACN)
- Decrease retention by *increasing* polarity of mobile phase (*i.e.*, increase **H_2O** in mobile phase to decrease retention)

Typical Elution Order for Test Compounds on HILIC Column – RPLC Column Test Mixture



Column	4.6 x 150 mm
Mobile phase	75% ACN: 25% H2O
Flow Rate	0.5 ml/ml
Col Temp	25 °C
Injection Volume	5 µl

1. Notice Uracil
   - •normally unretained in RPLC
   - •retained in HILIC

2. Notice completely different retention order than in RPLC

How Does HILIC Work on Silica Based Columns?

A water layer must be adsorbed onto the stationary phase.

The polar analyte partitions into and out of this adsorbed layer.

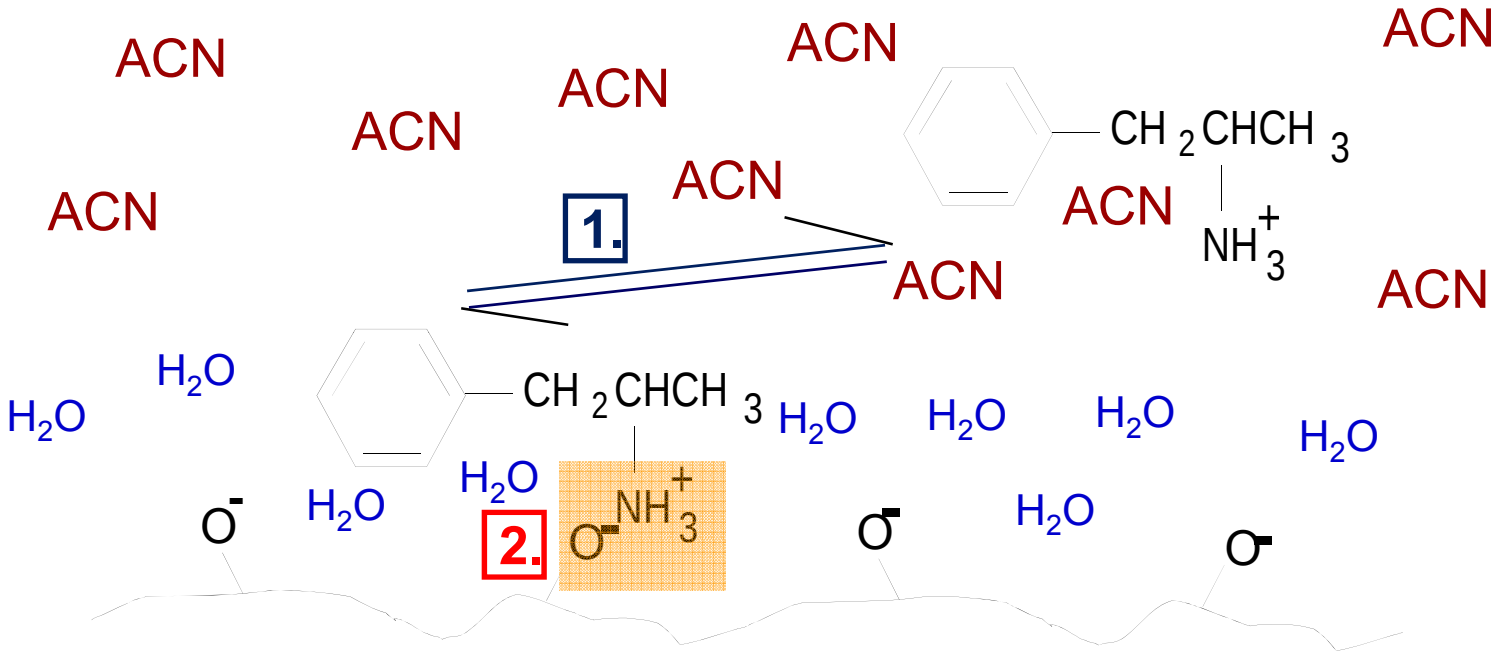
A charged polar analyte can also undergo ion exchange with the charged silica molecules (*i.e.*, cation exchange with amines)

The combination of these mechanisms drives retention in HILIC.

Retention/elution is from least to most polar – the opposite of reversed-phase LC.

HILIC offers more retention than reversed-phase for very polar bases.

How Does HILIC Work on Silica Based Columns? Potential Mechanisms

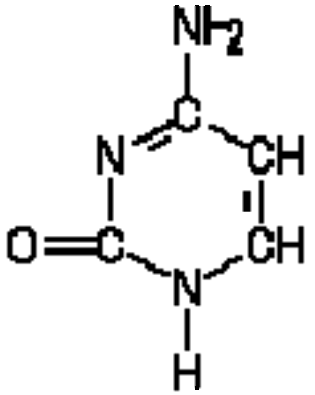


1. Partitioning in and out of adsorbed water layer
2. Ion exchange with silanols

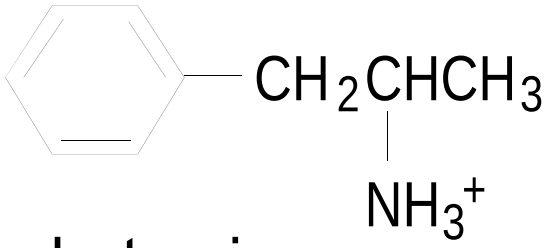
Examples

Some Typical Analytes for HILIC

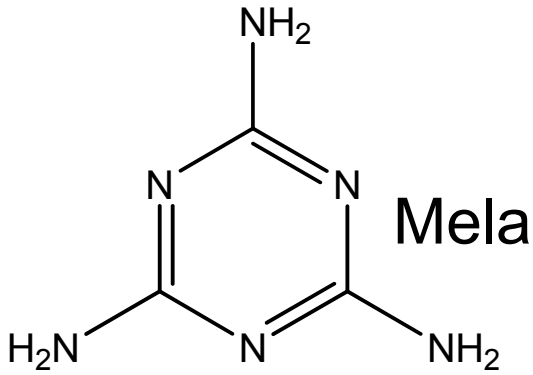
- Amino acids (when only a few are of interest)
- Nucleobases (purines and pyrimidines,
  adenine, guanine, thymine,
  thymine, cytosine, uracil)
- Nucleosides (Adenosine, cytosine etc.)
- Alkaloids
- Carbohydrates
- Polar compounds, small basic compounds



Cytosine



Amphetamine

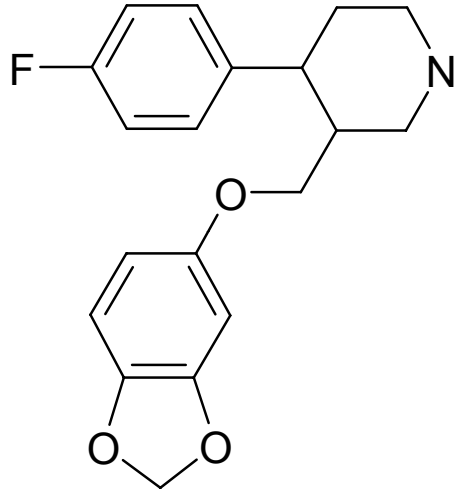


Melamine

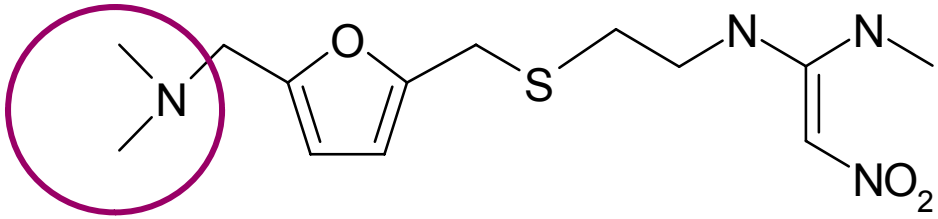
Comparison of Reversed-Phase and HILIC

Structures of Drug Compounds Studied

a) Paroxetine
Antidepressant
MW 329.36



b) Ranitidine
Antiulcerative
MW 314.41



Basic portion of molecule, impacts HILIC retention

Chromatographic Conditions

RPLC

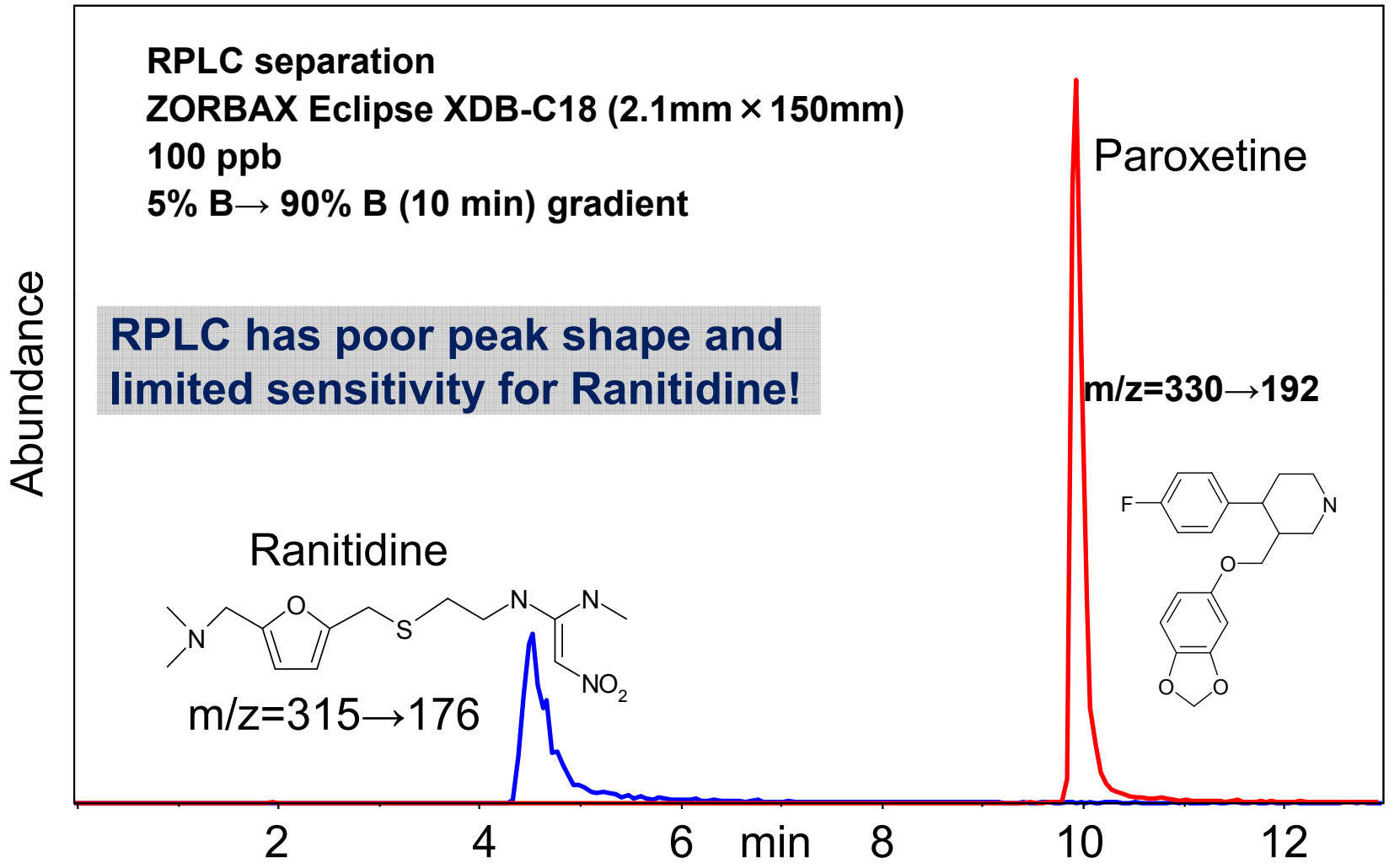
Instrument:	**Agilent Series 1100 HPLC**
Column:	**ZORBAX Eclipse XDB-C18 (2.1 mm × 150 mm, 5 µm)**
Mobile phase:	A: **8-mM HCO_2NH_4 in water**; B: **8-mM HCO_2NH_4 in 95% ACN/5% water**
Gradient:	**5% B to 90% B in 10 min**
Column temp:	**40 °C**
Sample volume:	**5 µL**
Flow rate:	**0.3 mL/min**

HILIC

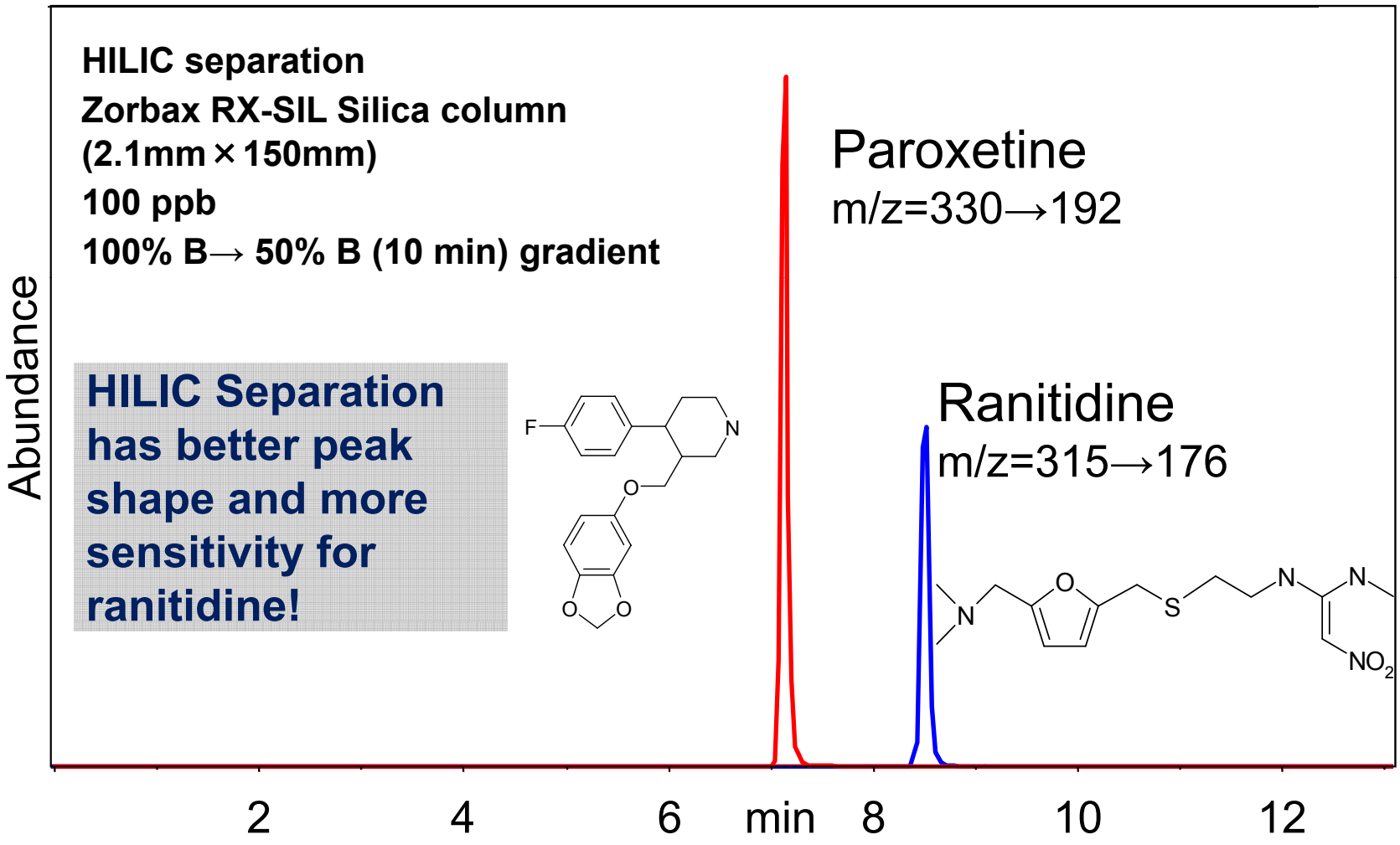
Everything same as for RPLC except for column and gradient conditions:

Column:	**ZORBAX Rx-SIL (2.1 mm × 150 mm, 5 µm)**
Gradient:	**100% B to 50% B in 10 min**

LC/MS/MS Separation of Paroxetine & Ranitidine on ZORBAX Eclipse XDB-C18 Column (RPLC Mode)

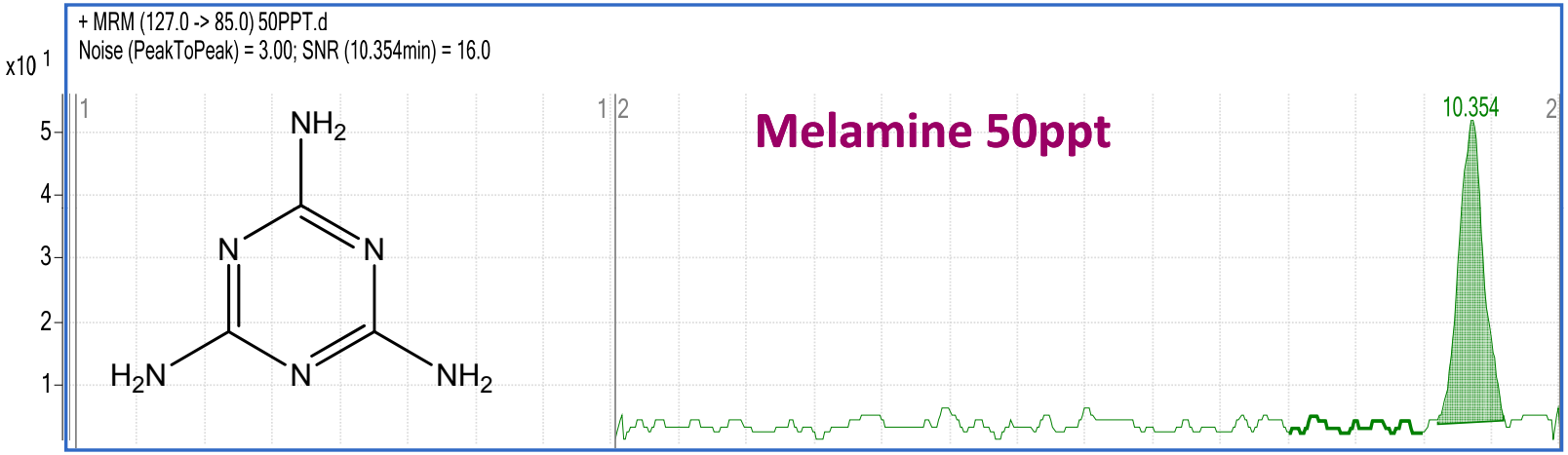


LC/MS/MS Separation of Paroxetine and Ranitidine on ZORBAX Rx-SIL Column (HILIC Mode)- 100 ppb Level



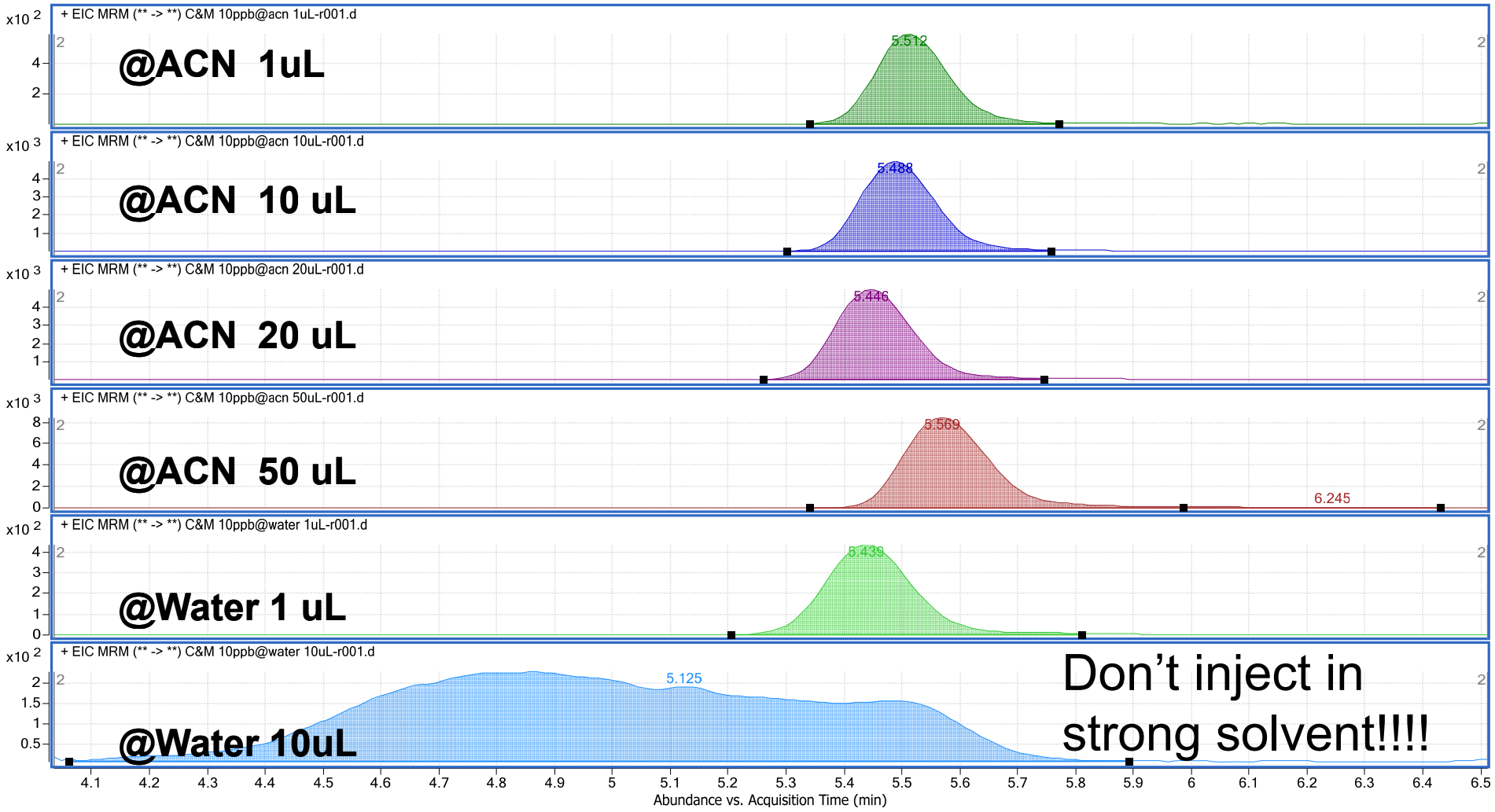
HILIC Separations are Ideal for LC/MS Analysis of Melamine

HILIC Separation Using ZORBAX Rx-Sil



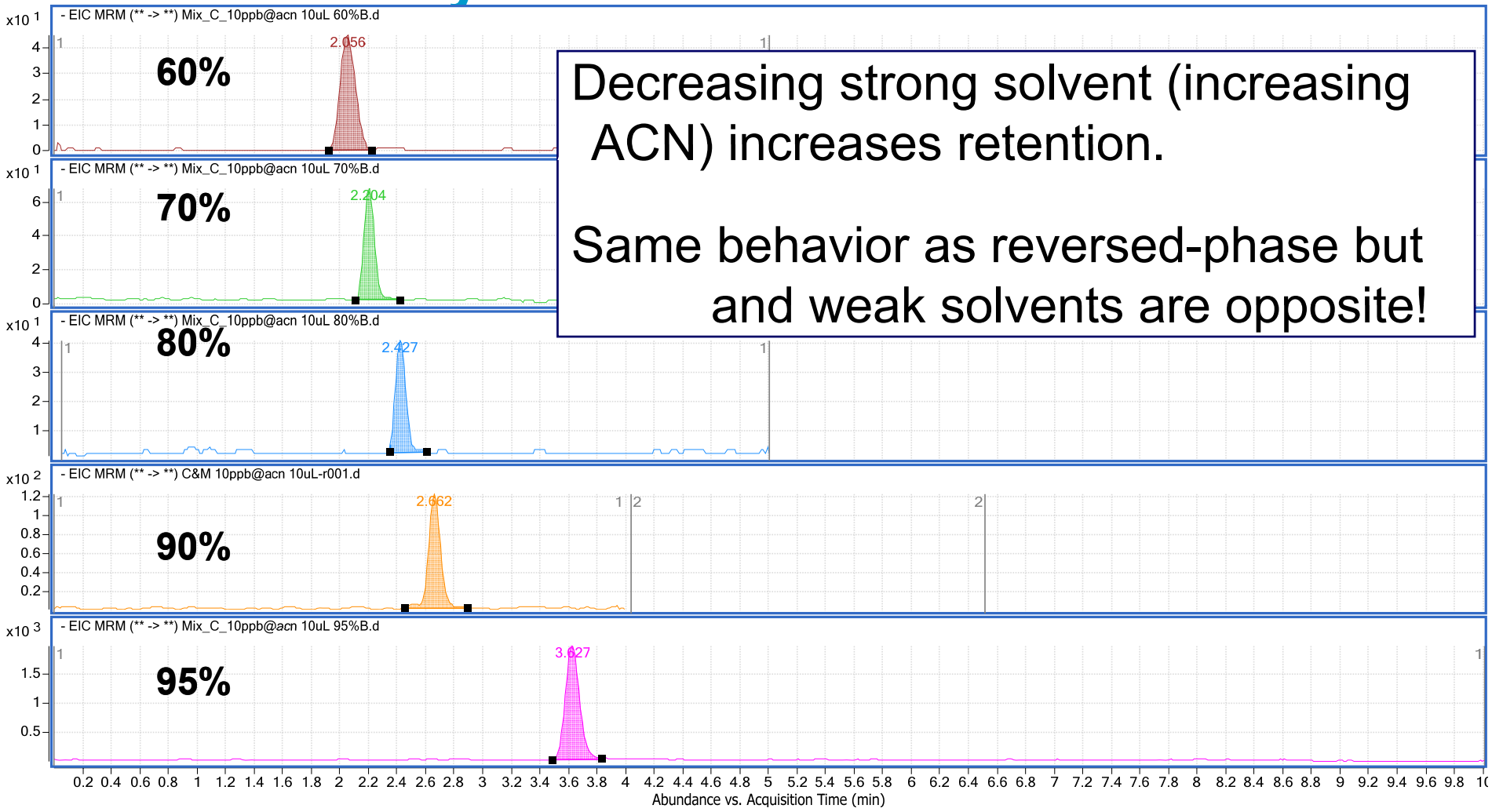
HPLC system	**: Agilent 1200 RRLC**
Column	**: Agilent ZORBAX Rx-Sil, 2.1×150 mm, 5 um**
Injection Volume	**: 10 μL**
Temp	**: 40℃**
Flow rate	**: 0.2 mL/min**
Mobile phase	**: A - 5 mM Ammonium acetate in Water**
	: B - 5 mM Ammonium acetate in ACN
Isocratic	**: 95%B**

Effect of Injection Solvent & Volume on Melamine Retention



Mobile Phase

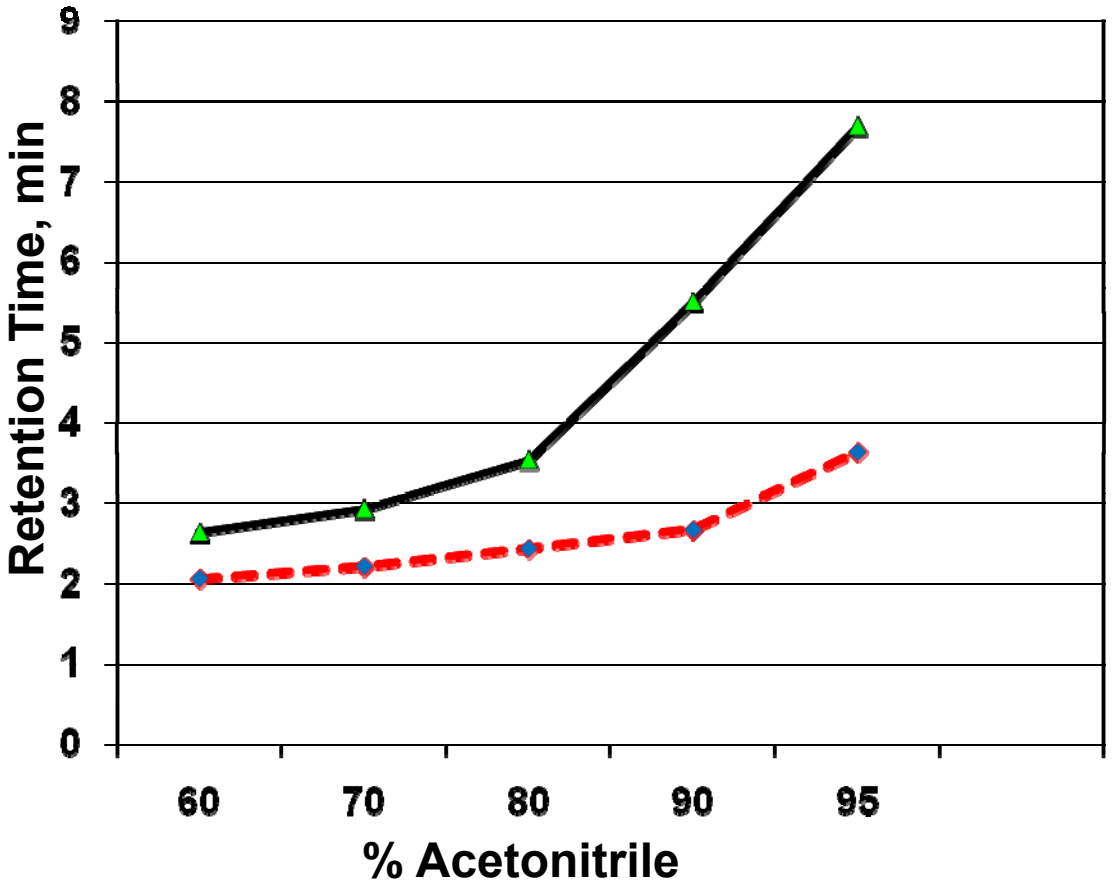
Effect of Increasing % ACN on Cyanuric Acid Retention



Decreasing strong solvent (increasing ACN) increases retention.

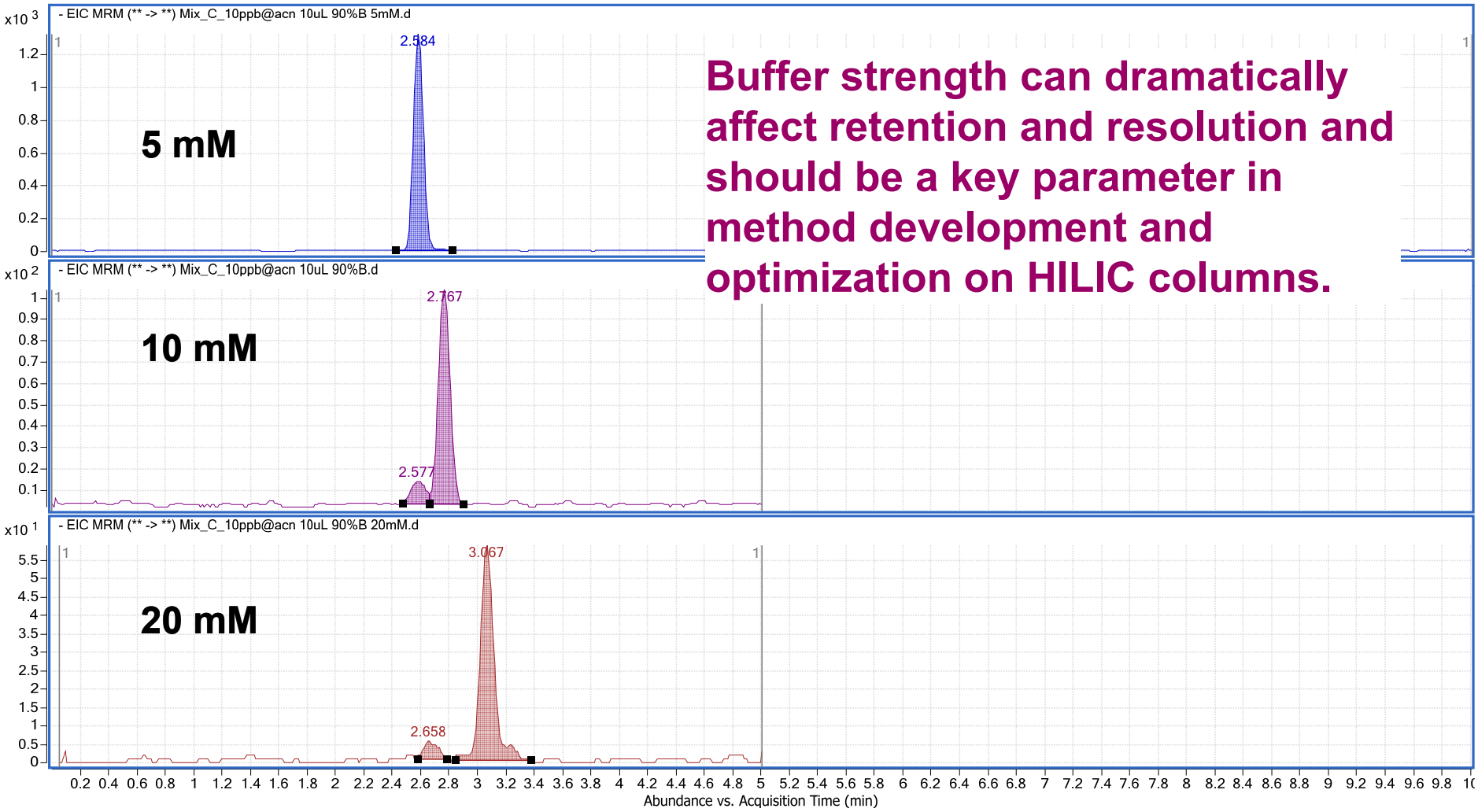
Same behavior as reversed-phase but and weak solvents are opposite!

Retention Time Changes with Changes in Mobile Phase Composition



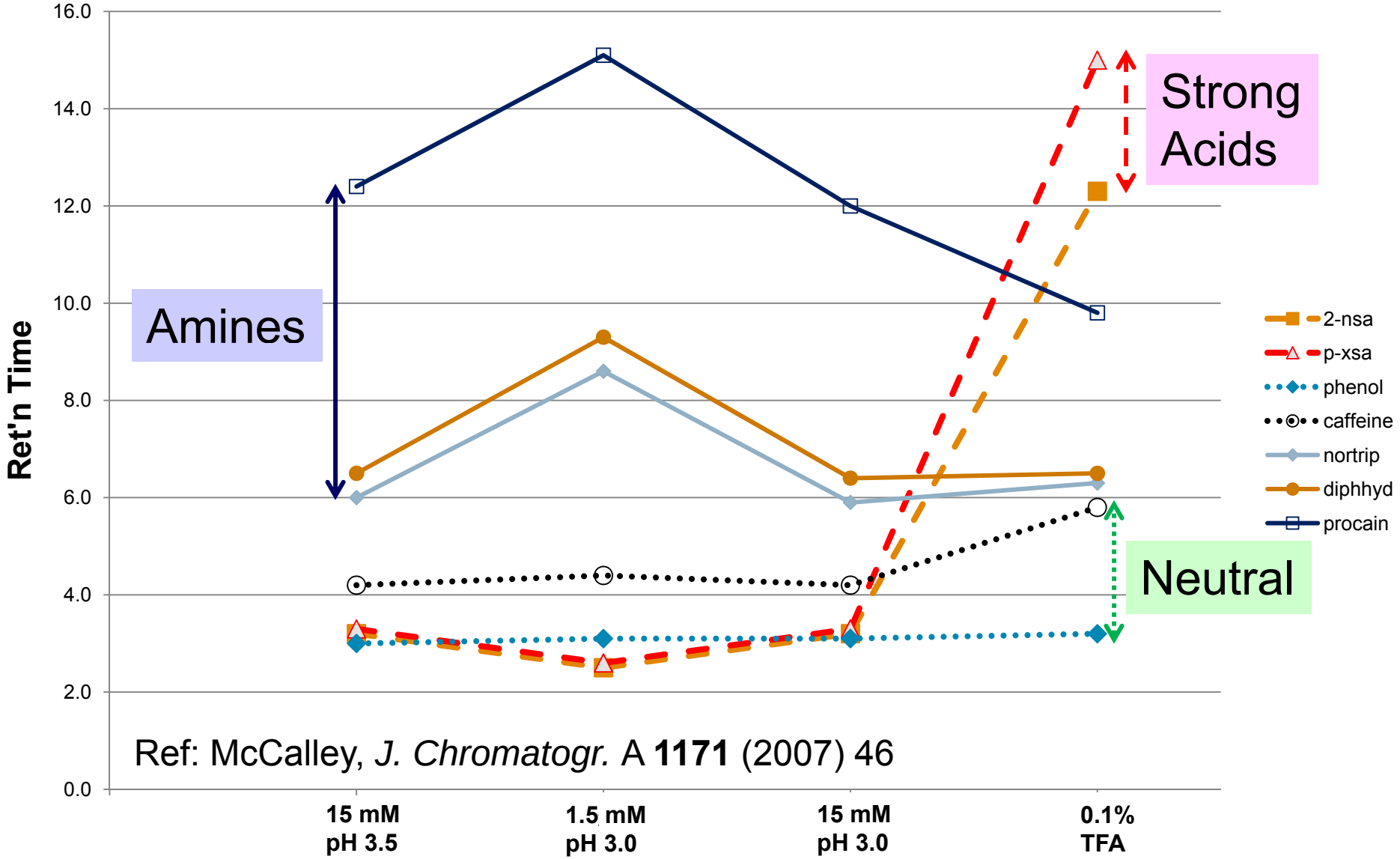
Mobile phase:
A= 5 mM NH_4OAc/H20
B= 5 mM NH_4OAc/ACN

Effect of Ammonium Acetate Concentration on Cyanuric Acid Retention



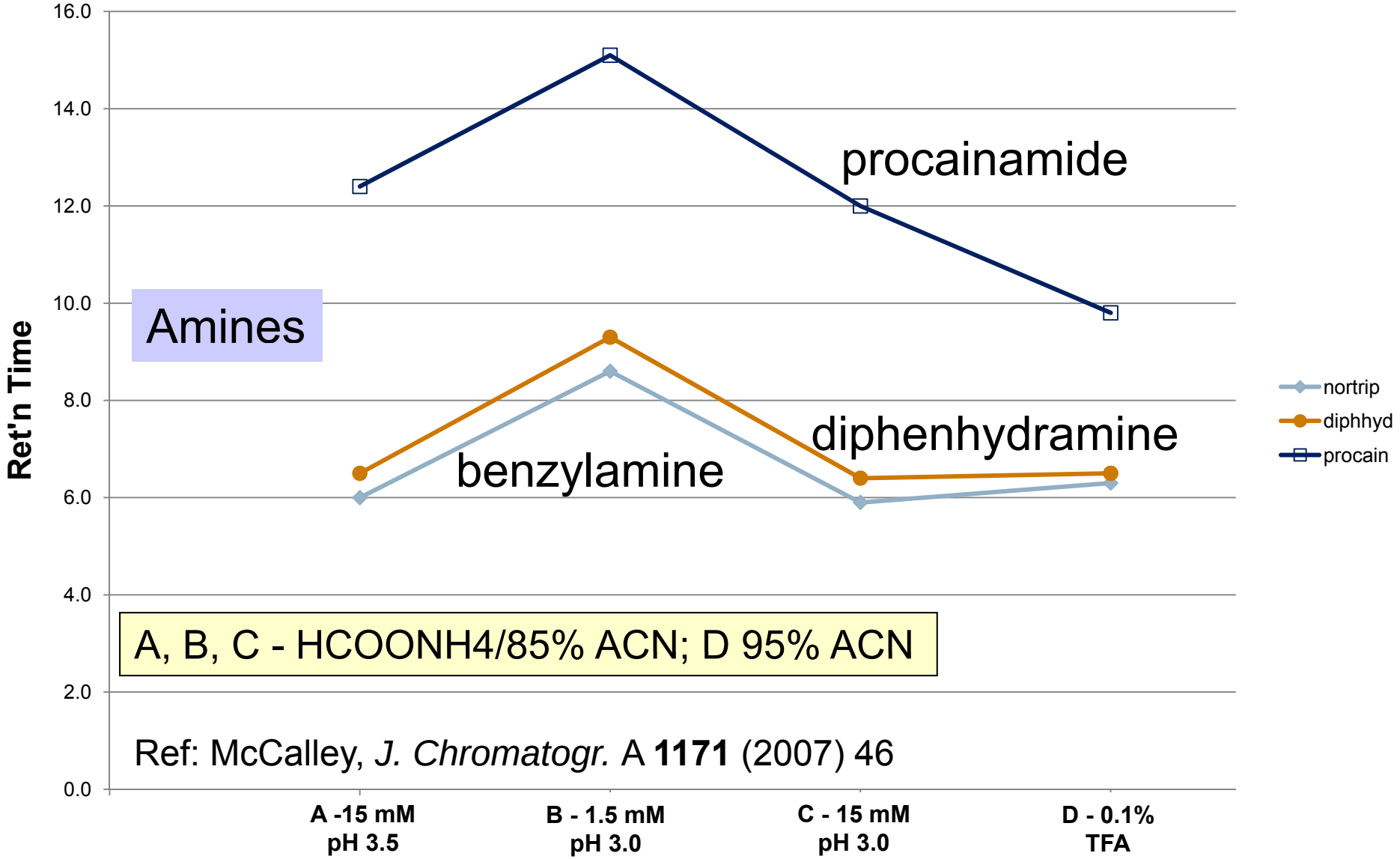
Buffer strength can dramatically affect retention and resolution and should be a key parameter in method development and optimization on HILIC columns.

Effect of pH, Buffer on Retention on Silica

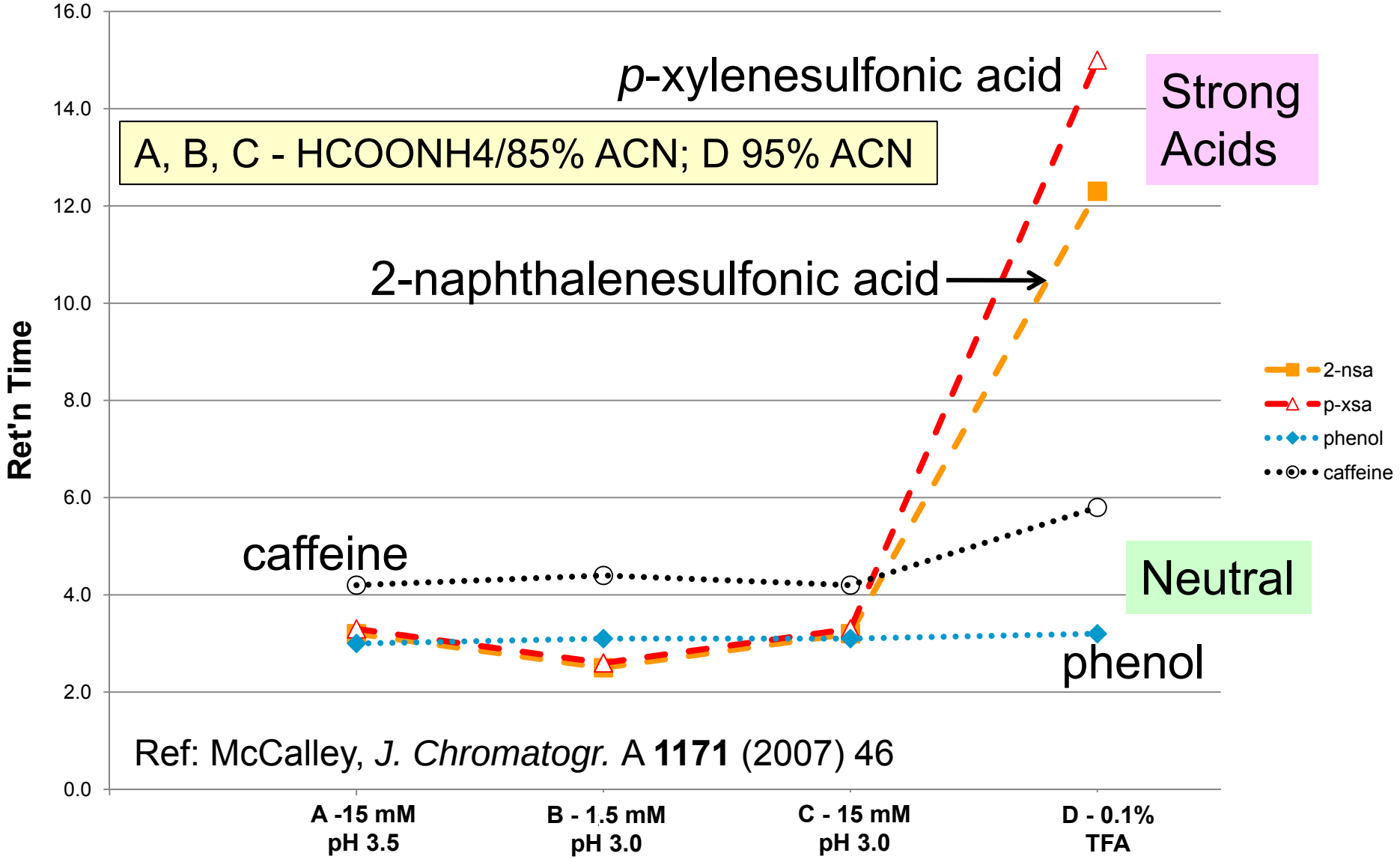


Ref: McCalley, *J. Chromatogr.* A **1171** (2007) 46

Effect of pH, Buffer on Retention on Silica



Effect of pH, Buffer on Retention on Silica



Effect of Buffer, pH

- **Controls Ionization of Silica**
- **Controls Ionization of Analyte**
- **Organic solvent affects the actual [H^+]**
- **Acids can be repulsed (reduces ret'n time) by anionic silica**
- **Buffer can mask anionic sites (reduce ret'n time of amines, increase for acids)**

Effect of Temperature

- **Higher temperature – improves kinetics**

  sharper peaks, shorter ret'n times

- **Lower temperature – improves selectivity**

- **Affects buffer equilibrium (effective pH)**

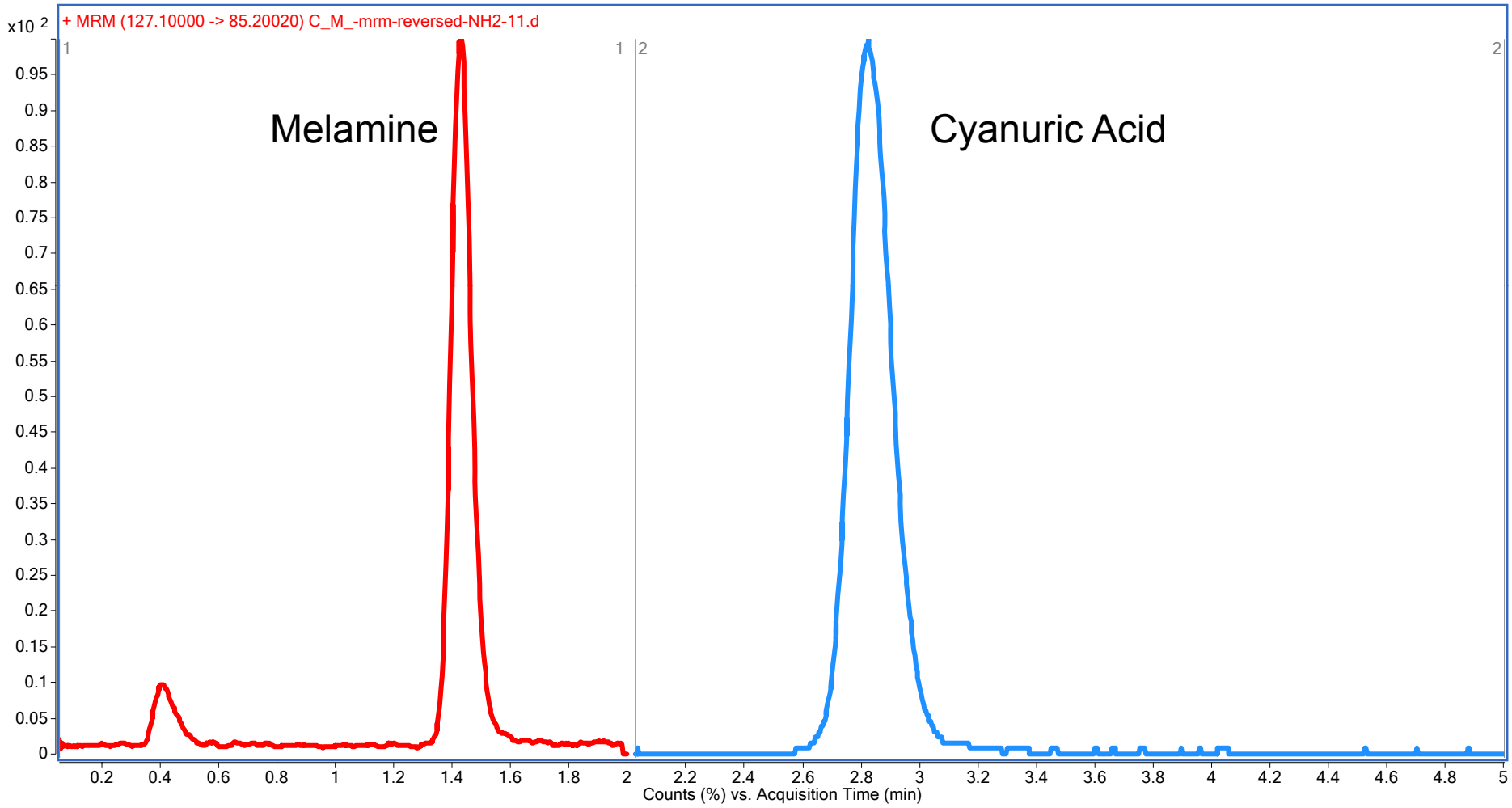
Columns

Comparison of Silica and Amino HILIC Columns

HPLC system	Agilent 1200
Column 1	Agilent Zorbax-NH2, 2.1×50 mm, 5 um
Column 2	Agilent Zorbax-Rx Sil, 2.1×150 mm, 5 um
Injection Volumn	2 uL
Temp	25℃
Flow rate	0.4 mL/min
Mobile phase	A - 5 mM Ammonium acetate in Water
	B - 5 mM Ammonium acetate in ACN
Isocratic	95%B

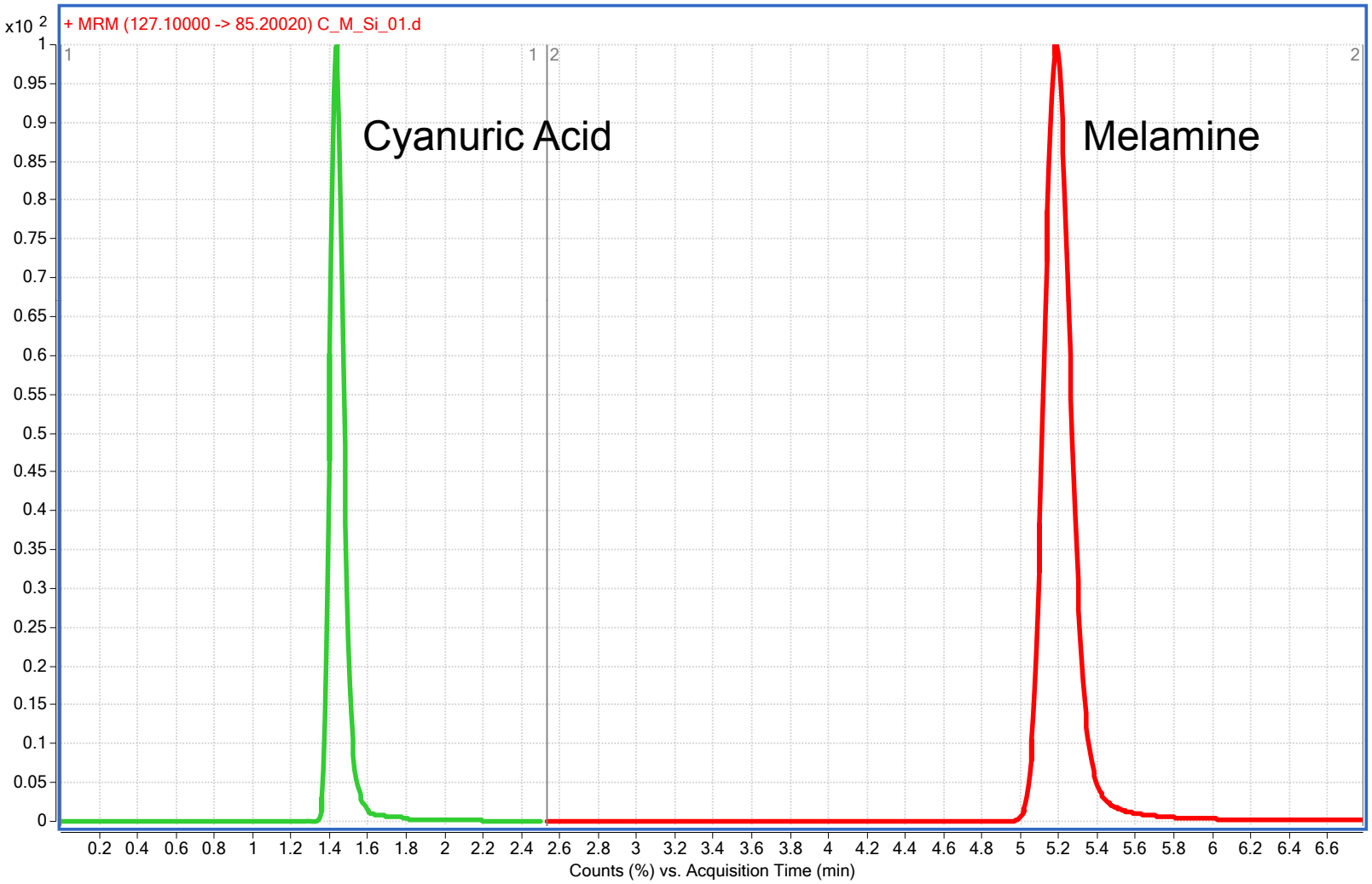
HILIC Separation Using Zorbax NH_2

5 mM Ammonium acetate in 5% H_2O/ACN



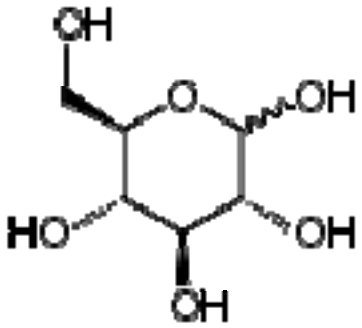
HILIC Separation Using Zorbax Rx-Sil

5 mM Ammonium acetate in 5% H_2O/ACN

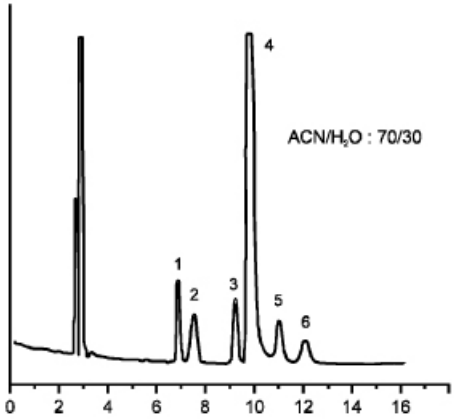


HILIC Separation of Sugars Using Zorbax Rx-NH_2

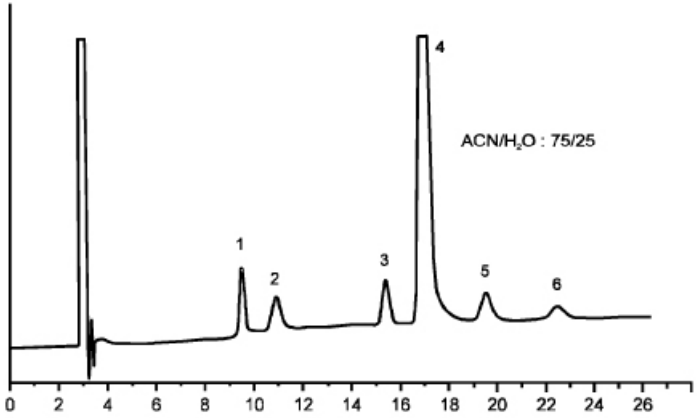
Comparison 70/30 and 75/25 ACN/H_2O



glucose



1. Fructose
2. Glucose
3. Saccharose
4. Palatinose
5. Trehalulose
6. Isomaltose



ZORBAX NH_2 (4.6 x 250 mm) (Agilent Part No. 880952-708)
Mobile Phase: ACN : H_2O, as indicated
1 mL/min, Detect. = Refractive Index

Method Development

Method Development/Optimization

Systematic Approach to Method Development

- **Stationary phase**
- **Mobile Phase**
- **Buffer, Buffer concentration**
- **pH**

Method Development/Optimization

Systematic Approach to Method Development

- **Separations are too complicated to expect to find optimum conditions using "Random Walk"**
- **Investigate effects of buffer, pH, buffer concentration**
- **Recommend use of experimental design**

cf: B. Dejaegher, D. Mangelings, Y. Vander Heyden, *J. Sep. Sci.*, **31** (2008) 1438

Method Development/Optimization

Starting Conditions

- **Silica**
- **Ammonium formate, 5, 10, 20 mM**
- **pH 3.0, 3.5, 4.0 (5, 6 ?)**
- **ACN/H_2O 97%, 95%, 90%, 85%, 75%**
- **TFA?**

Typical Stationary Phases - Polar

- **Silica**
- **Amine**
- **Diol**
- **Amide**
- **Zwitterionic**

Elution Order Inversely Related to RPLC, BUT Not Opposite

- **Mechanism, Retention is not exactly opposite of RPLC**
- **RPLC cannot be used as predictive model**
- **Not truly "orthogonal", but dissimilar separation, complementary technique**
- **Acids often poorly retained**

Summary - Advantages of HILIC

Retention of polar compounds

Good peak shape for basic compounds where RPC may give tailing and/or low efficiency

Higher flow rates and/or long columns can be used due to low viscosity mobile phases with high organic content; greater efficiency

Enhanced detection sensitivity with MS

- Efficient spraying and de-solvation in electrospray MS
- As much as 3X sensitivity

Can directly inject extracts from C18 SPE cartridges or acetonitrile precipitated plasma supernatant

Dissimilar to RPLC (2D separations); elution order reversal, Complementary separations

References

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